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# Further Characterization of L-Methionine $\gamma$ -Lyase and Enzymatic Synthesis of Sulfur Amino Acids

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CITATION:

Esaki, Nobuyoshi ...[et al]. Further Characterization of L-Methionine  $\gamma$ -Lyase and Enzymatic Synthesis of Sulfur Amino Acids. Bulletin of the Institute for Chemical Research, Kyoto University 1982, 59(5-6): 321-331

ISSUE DATE:

1982-03-15

URL:

<http://hdl.handle.net/2433/76962>

RIGHT:

## Further Characterization of L-Methionine $\gamma$ -Lyase and Enzymatic Synthesis of Sulfur Amino Acids\*

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Received April 7, 1980

L-Methionine  $\gamma$ -lyase is distributed widely in *Pseudomonas* species, and *Ps. putida* (IFO 3738) shows the highest activity of enzyme, which was inducibly formed by L-methionine. The enzyme catalyzes  $\alpha$ ,  $\gamma$ - and  $\alpha$ ,  $\beta$ -elimination reactions of L-methionine and S-methyl-L-cysteine and their derivatives, and also  $\gamma$ - and  $\beta$ -replacement reactions of these amino acids with various thiols to produce the corresponding S-substituted homocysteines and S-substituted cysteines, respectively. The replacement reactions facilitate the synthesis of various sulfur amino acids.

KEY WORDS: Pyridoxal-phosphate enzyme/ L-Methionine  $\gamma$ -lyase/  
Elimination reaction/ Replacement reaction/ Sulfur  
amino acids/ Enzymatic synthesis/

### INTRODUCTION

L-Methionine  $\gamma$ -lyase (EC 4.4.1.11) catalyzes the conversion of L-methionine into methanethiol,  $\alpha$ -ketobutyrate and ammonia and requires pyridoxal phosphate (pyridoxal-P) as a coenzyme. We have purified the enzyme to homogeneity from *Pseudomonas putida* (IFO 3738) and characterized its properties.<sup>1,2)</sup>

In this paper, we describe further studies on the enzymological properties of L-methionine  $\gamma$ -lyase and enzymatic synthesis of sulfur amino acids by  $\gamma$ - and  $\beta$ -replacement reactions.

### EXPERIMENTAL

**Materials.** L-Methionine and other amino acids were obtained from Ajinomoto Co., thiols were from Tokyo Chemical Co., pyridoxal-P was from Kyowa Hakko Kogyo, 3-methyl-2-benzothiazolonehydrazone hydrochloride was from Aldrich Chemical Co. DL-Vinylglycine was kindly provided by Dr. Walsh in Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.<sup>3)</sup> The other chemicals were analytical grade reagents.

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\* This paper was accepted on the occasion of the retirement of Prof. emeritus T. Yamamoto, and was dedicated to him.

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*Microorganisms and Conditions of Culture.* *Pseudomonas putida* (IFO 3738) and other organisms were grown in a medium containing L-methionine as described previously.<sup>1)</sup> The harvested cells were washed twice with 0.85% NaCl solution and subsequently with 0.01 M potassium phosphate buffer (pH 7.2) containing  $10^{-5}$  M pyridoxal-P and 0.01% 2-mercaptoethanol. The washed cells were stored frozen at  $-20^{\circ}\text{C}$  until used.

*Enzyme Preparation.* The enzyme was purified to homogeneity from a cell-free extract of *Ps. putida* (IFO 3738) as described previously.<sup>1)</sup>

*Enzyme Assay.* The enzymatic  $\alpha$ ,  $\gamma$ -elimination reaction was routinely followed by determining  $\alpha$ -ketobutyrate formed (Method A)<sup>2)</sup> For the replacement reaction, the enzyme activity was determined by measuring the amount of sulfur amino acids formed with ninhydrin after separation by paper chromatography (Method B).<sup>2)</sup> Protein was determined by the method of Lowry *et al.*<sup>4)</sup> (4) with crystalline egg albumin as a standard. Concentrations of the purified enzyme were derived from the absorbance at 278 nm ( $A_{1\text{cm}}^{1\%}=6.58$ ).<sup>2)</sup> One unit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1  $\mu\text{mol}$  of  $\alpha$ -ketobutyrate per min. The specific activity is expressed as units per mg of enzyme.

*Other Analytical Method.* Spectrophotometric measurements were made with a Shimadzu MPS-50L recording spectrophotometer or with a Carl Zeiss PMQ II spectrophotometer with a 1-cm light path. Amino acids in the incubation mixture were identified by chromatography with authentic materials on an amino acid analyzer (Yanagimoto LC-5S) by the method of Spackman *et al.* as described previously.<sup>2)</sup> Sulfur amino acids on a paper chromatogram were visualized with a platinum reagent.<sup>5)</sup> Infrared spectra were taken with a Hitachi EPI-S2 spectrophotometer and proton magnetic resonance spectra with a Varian Associate recording spectrometer (A 60) at 60 MHz in deuterium oxide and deuteriotrifluoroacetic acid with sodium 4, 4-dimethyl-4-silapentane-5-sulfonate and tetramethylsilane, respectively, as an internal standard. Chemical shifts are reported in  $\delta$  values (parts per million).

## RESULTS

*Distribution of L-Methionine  $\gamma$ -Lyase.* The activity of L-methionine  $\gamma$ -lyase in various strains of bacteria was investigated by measuring the formation of  $\alpha$ -ketobutyrate with the crude enzyme. *Ps. putida* (IFO 3738) in which L-methionine  $\gamma$ -lyase occurs most abundantly was chosen for the purpose of purification of the enzyme, though the considerably high activity was found also in *Ps. taetrolens*, *Ps. putida* (IFO 12995) and *Ps. desmolytica* (Table I).

*Effect of Added L-Methionine in the Growth Medium on Enzyme Activity.*

Table II shows data concerning the inducibility of L-methionine  $\gamma$ -lyase. Cells grown in the medium containing peptone and glycerol without methionine yielded

# L-Methionine $\gamma$ -lyase

Table I. Bacterial Distribution of L-Methionine  $\gamma$ -Lyase<sup>a)</sup>

Strains	Sp. Act.	
	I	II
<i>Enterobacter cloacae</i> (IFO 3320)	0.049	0.001
<i>Pseudomonas taetrolens</i> (IFO 3460)	0.018	0.005
<i>putida</i> (IFO 3738)	0.023	0.020
<i>putida</i> (IFO 12996)	0.007	0.009
<i>desmolytica</i> (IFO 12570)	0.033	0.010

a) The conditions of culture is given in Experimental Procedure. The washed cells were disrupted by grinding in a mortar with levigated aluminum oxide and extracted with 0.01 M potassium phosphate buffer (pH 7.2) containing  $10^{-5}$ M pyridoxal-P and 0.01% 2-mercaptoethanol. The supernatant solution obtained by centrifugation was dialyzed at 4°C for 20 h against the same buffer and employed as the crude enzyme. Enzyme activity was measured by Method A under aerobic (I) or anaerobic (II) conditions. The anaerobic incubation was carried out in a Thunberg test tube, the air in which was replaced by N<sub>2</sub> gas. Of the organisms capable of deaminating L-methionine under aerobic conditions, following strains were found unable to deaminate L-methionine under anaerobic conditions: *Escherichia coli* 26T (ICR B-0029), *E. coli* E9 (ICR B-0040), *Proteus mirabilis* (IFO 3849), *Alcaligenes faecalis* (IAM B-141-1), *Bacillus subtilis* (IAF 1193), *Agrobacterium radiobacter* (IAM 1526), *Hafnia alvei* (IFO 3731), *Brevibacterium ammoniagenes* (IFO 12072), *Pseudomonas reptilivora* (IFO 3461), *Ps. aeruginosa* (IFO 3924), *Ps. aeruginosa* (IFO 3918) and *Ps. aeruginosa* (IFO 3899).

Table II. Effect of Added L-Methionine in the Medium on the Enzyme Activity<sup>a)</sup>

Medium (addition %)	Growth (absorbance at 610 nm)	Specific Activity	Total Units
L-Methionine 0.1	0.583	0.019	2.81
0.2	0.481	0.032	3.08
0.3	0.339	0.045	3.07
0.4	0.315	0.045	3.05
0.5	0.292	0.045	2.98
None	0.385	0.014	1.21

a) The growth medium contained, in addition to the indicated concentrations of L-methionine, 0.1% peptone, 0.2% glycerol, 0.1% KH<sub>2</sub>PO<sub>4</sub>, 0.1% K<sub>2</sub>HPO<sub>4</sub>, 0.01% MgSO<sub>4</sub>·7H<sub>2</sub>O and 0.01% yeast extract. The organisms were grown and the activity of the dialyzed crude enzyme was determined in the method described previously.<sup>2)</sup>

minimally detectable enzyme activity. Addition of L-methionine at an initial concentration of 0.3% resulted in a 2.5-fold increase in activity.

## Change in L-Methionine $\gamma$ -Lyase Activity during the Cell Growth.

Relationship between L-methionine  $\gamma$ -lyase activity and cultivation time is shown in Figure 1. The total activity was high in the stationary phase, in particular 18 h after initiation of growth, and then decreased gradually.

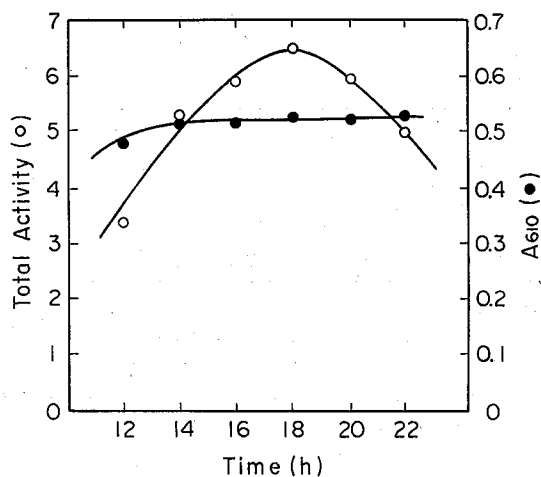


Fig. 1. Relationship between the enzyme activity and bacterial growth time. The composition of the medium is described in Experimental. Growth of the cells was followed by measurement of the absorbance at 610 nm.

Table III. Substrate Specificity

Substrate	Relative Activity
L-Methionine	100
D-Methionine	0
$\alpha$ -Methyl-L-methionine	0
N-Acetyl-L-methionine	0
L-Ethionine	90
L-Trifluoromethionine	180
L-Homocysteine	71
DL-Methionine sulfone	87
DL-Methionine sulfoxide	35
L-Methionine-DL-sulfoximine	27
S-Methyl-L-methionine	8
L-Norleucine	0
L-Norvaline	0
L- $\alpha$ -Aminobutyrate	0
S-Methyl-L-cysteine	20
S-Ethyl-L-cysteine	24
L-Cysteine	11
S-Carboxymethyl-L-cysteine	0
S-( $\beta$ -Aminoethyl)-L-cysteine	14
L-Cystine	0
L-Cystathionine	0
L-Serine	0
L-Homoserine	0

**Substrate Specificity.** The ability of L-methionine  $\gamma$ -lyase to catalyze elimination reaction of various amino acids is presented in Table III. In addition to L-methionine, several derivatives of L-methionine and L-cysteine, *e.g.*, DL-selenomethionine, L-trifluoromethionine, L-ethionine, DL-methionine sulfone, L-homocysteine, and S-ethyl-L-cysteine serve as the effective substrates. S-Methyl-L-methionine, S-( $\beta$ -aminoethyl) L-cysteine, L-cysteine can be decomposed, though slowly, whereas D-methionine,  $\alpha$ -methyl-L-methionine, N-acetyl-L-methionine, L-cystathionine, L-cystine, L-norvaline, L-norleucine, L-homoserine are inert. These results provide evidence that the enzyme catalyzes both  $\alpha$ ,  $\gamma$ - and  $\alpha$ ,  $\beta$ -elimination reactions.

**Effect of Temperature.** When the  $\alpha$ ,  $\gamma$ -elimination reaction of L-methionine was performed at the various temperature, the maximum reaction velocity was obtained at 50–60°C (Figure 2). The reaction rate increased linearly when the temperature was raised in the range of 30–50°C. However, the velocity decreased rapidly over 60°C.

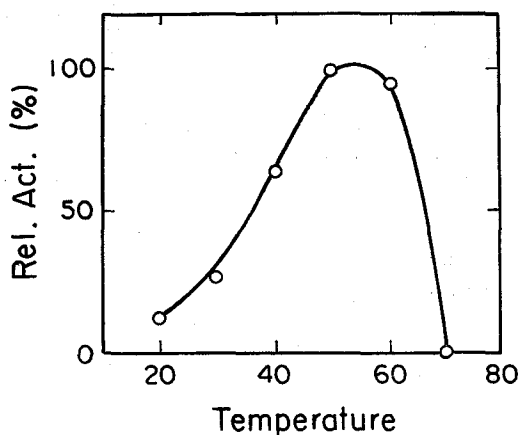


Fig. 2. Effect of temperature on  $\alpha$ ,  $\gamma$ -elimination of L-methionine. The reaction was performed at indicated temperature for 10 min in the reaction system described in Experimental.

**Effect of pH.** The enzyme has an optimum reactivity around pH 8.0 for  $\alpha$ ,  $\gamma$ -elimination of L-methionine when examined in the presence of Tris-maleate, potassium phosphate, Tris-HCl, borate, and glycine-KOH buffers (Figure 3). The reaction was inhibited approximately 50%, when Tris-HCl buffer was used.

**Inhibitors.** The various compounds were investigated for their inhibitory effects on enzyme activity (Table IV). The enzyme is inhibited most strongly by hydroxylamine, L-penicillamine and L-cycloserine, which are typical inhibitors for pyridoxal-P enzymes. D-Penicillamine and D-cycloserine are also inhibitory.

L-Methionine  $\gamma$ -lyase shows a high sensitivity to thiol reagents, *e.g.*, N-ethyl maleimide,  $\text{HgCl}_2$ , and *p*-chloromercuribenzoate.

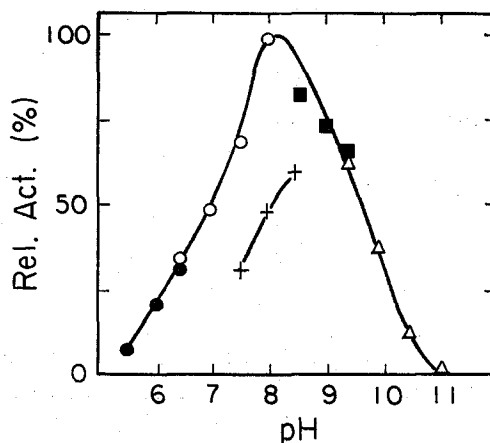


Fig. 3. Effect of pH on  $\alpha$ ,  $\gamma$ -elimination of L-methionine. The enzyme activity was determined with 3-methyl-2-benzothiazolonehydrazone (MBTH). The buffers used (final concentration; 0.05 M) are: (●), Tris-maleate; (○), potassium phosphate; (+), Tris-HCl; (■), sodium borate; (▲), glycine-KOH.

Table IV. Effect of Inhibitors on the Enzyme Activity<sup>a)</sup>

Compounds	Concentrations	Relative Activity
None		100%
L-Cycloserine	1 mM	26
D-cycloserine	1	91
L-Penicillamine	1	10
D-Penicillamine	1	76
NH <sub>2</sub> OH	0.1	0
NH <sub>2</sub> OH	0.01	46
N-Ethylmaleimide	1	0
PCMB <sup>b)</sup>	0.1	4
HgCl <sub>2</sub>	1	3
Iodoacetate	1	18
Iodoacetamide	1	11

a) The enzyme was incubated with inhibitors at 30°C for 10 min. The reactions were started by addition of L-Methionine.

b) PCMB, *p*-chloromercuribenzoate.

#### *Enzymatic Synthesis of Sulfur Amino Acids by Replacement Reactions.*

Several pyridoxal-P enzymes have multiple catalytic functions, *e.g.*, tryptophanase,<sup>6)</sup>  $\beta$ -tyrosinase<sup>7)</sup> and cystathionine  $\gamma$ -synthase,<sup>8)</sup> which catalyze both elimination and replacement reactions. We examined whether L-methionine  $\gamma$ -lyase catalyzes a replacement reaction as well. When methionine was incubated with the enzyme in the presence of ethanethiol, the formation of  $\alpha$ -ketobutyrate was markedly diminished as shown in Figure 4. The reaction mixture was paper-chromatographed

# L-Methionine $\gamma$ -lyase

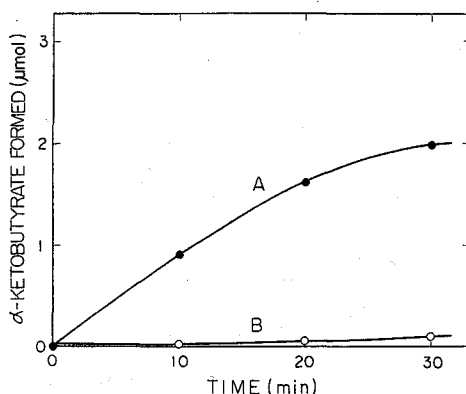


Fig. 4. Inhibition of  $\alpha$ -ketobutyrate formation by ethanethiol. Incubations were carried out at 30°C for various period. The reaction mixture contained 50  $\mu$ mol of L-methionine, 0.01  $\mu$ mol of pyridoxal-P, 100  $\mu$ mol of potassium phosphate buffer (pH 8.0), 40  $\mu$ mol of ethanethiol and enzyme in a final volume of 1.0 ml. The amount of  $\alpha$ -ketobutyrate formed was determined by Method A. Curve A, The reaction in the absence of ethanethiol; curve B, the reaction in the presence of ethanethiol.

with *n*-butanol-acetic acid-water (12: 3: 5, v/v/v) as the solvent system, and the spots were visualized with ninhydrin and the platinum reagent. In addition to methionine, a new sulfur-containing amino acid was observed. The product was isolated from the reaction mixture, and then identified as L-ethionine based on the physicochemical properties. L-Methionine was quantitatively converted into L-ethionine as a function of enzyme concentration and incubation time (Figure 5). Addition of enzyme and ethanethiol enhanced the velocity (Figure 6). We can synthesize L-ethionine enzymatically in a yield more than 95% under the good conditions. L-Ethionine was also produced by the  $\gamma$ -replacement reaction between the derivatives of methionine

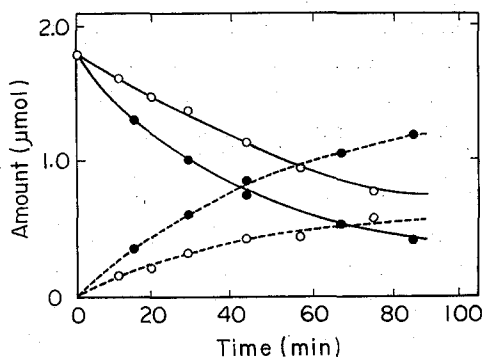


Fig. 5. Time course of ethionine synthesis. L-Methionine (—) and L-ethionine (---) were determined with ninhydrin as described in Experimental. Amount of the enzyme added to the reaction mixture are: (○), 0.02 unit; and (●), 0.04 unit.



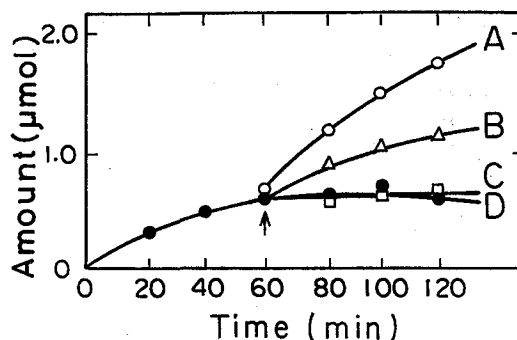


Fig. 6. Feeding effect of ethanethiol and the enzyme on ethionine synthesis. The reaction systems are described in Experimental. Curve A, 0.02 unit of the enzyme and 20  $\mu$ mol of ethanethiol; B, 20  $\mu$ mol of ethanethiol; C, 0.02 unit of the enzyme; and D, none were added after 60 min.

Table V. Relative Velocity of Synthesis of L-Ethionine and S-Ethyl-L-cysteine from Ethanethiol and Derivatives of Homocysteine and Cysteine by L-Methionine  $\gamma$ -Lyase

Type	Amino Acid Substrate	Relative Activity
$\gamma$ -Replacement (L-Ethionine Formation)	L-Methionine	100%
	DL-Methionine sulfone	22
	DL-Methionine sulfoxide	7
	L-Methionine-DL-sulfoximine	29
	L-Homocysteine	93
	S-( $\beta$ -Aminoethyl)-L-homocysteine	3
$\beta$ -Replacement (S-Ethyl-L-cysteine Formation)	S-Methyl-L-cysteine	73
	L-Cysteine	6

(*e.g.*, homocysteine, methionine sulfone and methionine sulfoxide), which are substrates for the elimination reaction, and ethanethiol (Table V).

When ethanethiol was replaced by various alkanethiols ( $C_3$ – $C_7$ ) and arylthio alcohols (benzenethiol and  $\beta$ -naphthalenethiol) in the reaction system, new sulfur amino acids corresponding to the substrates were synthesized enzymatically. Longer chain alkanethiols ( $C_8$ – $C_{10}$ ), however, were not substrates (Table VI). The amino acids produced from L-methionine and thiols were identified as the corresponding S-substituted homocysteines based on the proton magnetic resonance spectra and infrared spectra. The derivatives of ethanethiol, *e.g.*, 2-mercaptoethanol and cysteamine, also serve as the substituent donors to methionine. The products from 2-mercaptoethanol and cysteamine were identified as S-( $\beta$ -hydroxyethyl)homocysteine and S-( $\beta$ -aminoethyl)homocysteine, respectively. The relative rates of  $\gamma$ -replacement reaction of various substituted alkanethiols are listed in Table VII. Thiols with a charged group were generally poor substituent donors.

# L-Methionine $\gamma$ -lyase

Table VI. Relative velocity of synthesis of L-methionine related amino acids from L-methionine and thiols by L-methionine  $\gamma$ -lyase

RSH R=	Rel. Rate %	RSH R=	Rel. Rate %
Ethyl	100	<i>n</i> -Hexyl	17
<i>n</i> -Propyl	81	<i>tert</i> -Hexyl	5
<i>iso</i> -Propyl	38	<i>n</i> -Heptyl	4
<i>n</i> -Butyl	43	<i>tert</i> -Heptyl	0
<i>iso</i> -Butyl	75	<i>n</i> -Octyl	0
<i>sec</i> -Butyl	27	<i>tert</i> -Octyl	0
<i>tert</i> -Butyl	26	<i>tert</i> -Nonyl	0
<i>n</i> -Pentyl	43	<i>n</i> -Decyl	0
<i>iso</i> -Pentyl	52	Cyclohexyl	10
<i>sec</i> -Pentyl	12	Benzyl	71
<i>tert</i> -Pentyl	9	Phenyl	43
		$\beta$ -Naphthalene	6

Table VII. Relative velocity of synthesis of *S*-substituted homocysteines from L-methionine and thiols by L-methionine  $\gamma$ -lyase

RSH R=	Rel. Velocity %
-CH <sub>2</sub> CH <sub>3</sub>	100
-CH <sub>2</sub> COOH	11
-CH <sub>2</sub> COOCH <sub>2</sub> CH <sub>3</sub>	84
-CH <sub>2</sub> CH <sub>2</sub> OH	111
-CH <sub>2</sub> CH <sub>2</sub> COOH	7
-CH <sub>2</sub> CH <sub>2</sub> NH <sub>2</sub>	44
-CH <sub>2</sub> CH <sub>2</sub> NHCOCH <sub>3</sub>	267

Table VIII. Relative velocity of synthesis of *S*-substituted homocysteines from methionine or vinylglycine by L-methionine  $\gamma$ -lyase

RSH R=	Vinylglycine (%)	Methionine (%)
Ethyl-	100 (72%)	100 (100%)
<i>n</i> -Propyl	82	81
<i>n</i> -Hexyl	23	17
Benzyl	72	71

In addition, the enzyme catalyzes the  $\beta$ -replacement reaction between *S*-methyl-L-cysteine and ethanethiol to yield *S*-ethyl-L-cysteine (Table V).

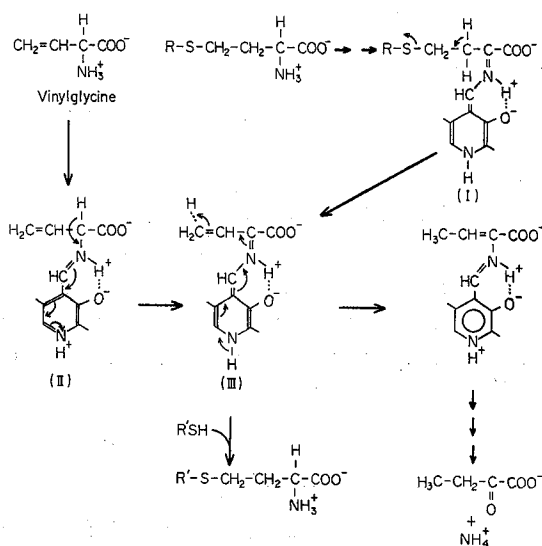
Vinylglycine is regarded as a common key intermediate of  $\alpha$ ,  $\gamma$ -elimination and  $\gamma$ -replacement reactions.<sup>9)</sup> L-Methionine  $\gamma$ -lyase catalyzes deamination reaction of vinylglycine to produce  $\alpha$ -ketobutyrate and ammonia.<sup>10)</sup> The enzyme catalyzes also  $\gamma$ -addition reaction of various alkanethiols to vinylglycine to yield the corresponding *S*-substituted homocysteines.<sup>10)</sup> Relative activity of the enzyme for various alkanethiols in  $\gamma$ -addition reaction of vinylglycine is close to that in  $\gamma$ -replacement reaction of methionine (Table VIII).

## DISCUSSION

The studies described here deal with the microbial distribution of L-methionine  $\gamma$ -lyase and the enzymological characterization of the enzyme. The high activity

was found in *Pseudomonas* species. *Ps. putida* (IFO 3738) in which L-methionine  $\gamma$ -lyase occurs most abundantly was chosen for the purpose of purification of the enzyme. The formation of the enzyme was induced by L-methionine in the growth medium.

The enzyme can decompose various  $\beta$ - and  $\gamma$ -substituted amino acids in addition to L-methionine. The enzyme also catalyzes the replacement reaction between the thiomethyl group of methionine and various thiols to yield the corresponding S-substituted homocysteines. In addition, the enzymatic  $\beta$ -replacement reaction occurs. Cystathionine  $\gamma$ -synthase of *Salmonella* also catalyzes  $\alpha$ ,  $\gamma$ -elimination and  $\gamma$ -replacement reactions of amino acids substituted at  $\gamma$ -C atom, and also  $\alpha$ ,  $\beta$ -elimination and  $\beta$ -replacement reactions of  $\beta$ -substituted amino acids.<sup>8)</sup> The mechanism of  $\alpha$ ,  $\beta$ -elimination and  $\beta$ -replacement reactions of pyridoxal-P enzymes was reviewed by Snell and Di Mari,<sup>11)</sup> and Davis and Metzler.<sup>9)</sup> The reaction proceeds through elimination of the  $\beta$ -substituent and then a common intermediate,  $\alpha$ -aminoacrylate Schiff base is formed. In  $\alpha$ ,  $\beta$ -elimination this intermediate is hydrolyzed to release ammonia and  $\alpha$ -keto acid and regenerate pyridoxal-P enzyme, whereas in  $\beta$ -replacement reaction nucleophilic ligand adds across the  $\alpha$ ,  $\beta$ -double bond to yield the product. For the  $\alpha$ ,  $\gamma$ -elimination and  $\gamma$ -replacement reactions, no model reaction has yet been presented. The proposed reaction mechanism for L-methionine  $\gamma$ -lyase is shown in Scheme 1. The formation of the first quinoid intermediate (I) is followed by a loss of a  $\beta$ -proton, which then eliminates the  $\gamma$ -substituent to form  $\beta$ ,  $\gamma$ -unsaturated compounds (III) (vinylglycine Schiff base). This intermediate is derived also from a pyridoxal-P aldimine intermediate of vinylglycine (II). The intermediate III can add a new  $\gamma$ -substituent, or tautomerizes to the  $\alpha$ ,  $\beta$ -unsaturated compound ( $\alpha$ -aminocrotonate Schiff base) and then decomposes, in nonenzymatic stage, to  $\alpha$ -ketobutyrate and ammonia. Reactivity of L-methionine  $\gamma$ -lyase toward vinylglycine presents an evidence for the proposed mechanism.



Scheme 1. Proposed reaction mechanism of L-methionine  $\gamma$ -lyase.

#### REFERENCES

- (1) H. Tanaka, N. Esaki, T. Yamamoto, and K. Soda, *FEBS Lett.*, **66**, 307 (1976).
- (2) H. Tanaka, N. Esaki, and K. Soda, *Biochemistry.*, **16**, 100 (1977).
- (3) J. Baldwin, S. Haber, C. Hoslins, and L. Kruse, *J. Org. Chem.*, **42**, 1239 (1977).
- (4) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- (5) G. Toennis and J. J. Kolb, *Anal. Chem.*, **23**, 823 (1951).
- (6) H. Yoshida, H. Kumagai, and H. Yamada, *Agric. Biol. Chem.*, **38**, 463 (1974).
- (7) H. KUMAGAI, H. Matsui, H. Ohgishi, K. Ogata, H. Yamada, T. Ueno, and H. Fukami, *Biochem. Biophys. Res. Commun.*, **34**, 266 (1969).
- (8) S. Guggenheim and M. Flavin, *J. Biol. Chem.*, **244**, 6217 (1969).
- (9) L. Davis and D. E. Metzler, *Enzymes*, 3rd Ed. **7**, 33 (1972).
- (10) N. Esaki, T. Suzuki, H. Tanaka, K. Soda, and R. R. Rando, *FEBS Lett.*, **84**, 309 (1977).
- (11) E. E. Snell and S. J. Di Mari, *Enzymes.*, 3rd Ed. **2**, 335 (1970).